Generation of normal and tumor organoids from human colorectal tissues

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Version 1.0 Version Date: 25 April 2022 Modifed by: Benardina Ndreshkjana

Introduction

Patient-derived tumor organoids (PDTOs) have emerged as excellent preclinical models. Extensive research shows that organoids complement animal models and offer unique possibility to study human diseases. They closely recapitulate the molecular and phenotypic characteristics of the parental tumor. Many culture protocols have been described for several cancer types. Organoid technology allows long-term expansions and they offer great advantage for studying drug sensitivities and resistances in medium-to-high-throughput screening.

Purpose

This standard operating procedure (SOP) describes the procedures of establishing organoids from primary human colorectal cancer tissues (CRC) and the respective healthy tissue samples.

Scope

This SOP provides a complete procedure for the establishment, expansion, freezing and thawing of the organoid cultures from colorectal tissue as well as the composition and preparation of the culture media.

1. Equipment, materials and chemicals

1.1 Equipment

- Cell culture incubator adjusted at 37 °C and 5 % CO2
- Centrifuge (Rotanta 460R, Hettich)
- Evos Fl microscope with 2X, 4X, 10X objectives
- Pipettes with sterile tips
- Pump + Aspiration pipettes
- Water bath or heating block
- Pipette boy

1.2 Materials

- 15- and 50-ml Falcons
- 5 ml Eppendorf tubes
- Sterile Petri dishes
- Sterile 12-well cell culture suspension plates (Greiner, #665102)
- Cryovials
- Sterile forceps
- Disposable scalpels
- 5, 10 and 25 ml pipets
- Freezing box

1.3 Chemicals

- Matrigel™ (Corning, #356231)
- Primocin (InvivoGen, #ant-pm-1)
- Y-27632 Rho kinase inhibitor (Biotrend, #Y5301)
- Recovery™ Cell Culture Freezing Medium (Thermo Fisher, #12648010)
- PBS (Thermo Fisher, # 10010015)

2. Medium recipes

2.1 Washing medium

Component	Concentration (stock)	Amount
DMEM (Thermo Fisher, #31966-047)	1x	500 ml
Bovine Albumin Fraction (Thermo Fisher, #15260-037)	7.5%	2.65 ml
Pent/Strep (Thermo Fisher, #15140-122)	100x	5 ml

2.2 Advanced DMEM+++

Component	Concentration (stock)	Amount
Advanced DMEM/F12 (Thermo Fisher, #12634-028)	1x	500 ml
Glutamax (Thermo Fisher, #35050038)	100x	5 ml
HEPES (Thermo Fisher, #15630056)	1M (100x)	5 ml
Pent/Strep (Thermo Fisher, #15140-122)	100x	5 ml

2.3 Medium for tumor organoid expansion

Component	Concentration (stock)	Amount
Advanced DMEM +++	1x	35 ml
*R-spondin (in house prepared CM)	1x	10 ml, 20% (v/v)
**Noggin (in house prepared CM)	1x	5 ml, 10% (v/v)
B-27 (Thermo Fisher, # 12587010)	50x	1 ml
N-acetylcysteine (Sigma-Aldrich, #A9165)	5M	125 µl
Epidermal growth factor (Peprotech, #AF-100-15)	500 µg/ml	5 µl
ALK5 inhibitor A83-01 (Tocris, #2939/10)	500 µM	50 µl
p38 inhibitor SB202190 (Sigma-Aldrich, #S7076)	30 mM	16.6 µl

**R-spondin1-conditioned medium was obtained from R-spondin producing cells (293T-HA-RspoI-Fc, from Calvin Kuo lab) and used under approved MTA.*

*** Noggin conditioned medium was obtained from Noggin producing cells (HEK293-mNoggin-Fc, from Hans Clevers lab) and used under approved MTA.*

2.4 Medium for normal organoid expansion

Component	Concentration (stock)	Amount
AdvD Advanced DMEM +++ (see 4.2.)	1x	25 ml
R-spondin (in house prepared CM)	1x	10 ml, 20% (v/v)
Noggin (in house prepared CM)	1x	5 ml, 10% (v/v)
Wnt surrogate-FC fusion protein (ImmunoPrecise, N001)	5 µM	3 µl (35 ng/µl)
B-27 (Thermo Fisher, # 12587010)	50x	1 ml
N-acetylcysteine (Sigma-Aldrich, #A9165)	5M	125 µl
Epidermal growth factor (Peprotech, #AF-100-15)	500 µg/ml	5 µl
ALK5 inhibitor A83-01 (Tocris, #2939/10)	500 µM	50 µl
p38 inhibitor SB202190 (Sigma-Aldrich, #S7076)	30 mM	16.6 µl

3. EDTA 4 mM

- EDTA (Thermo Fisher, #15575020), 500 mM, 400 µl
- *PBS (Thermo Fisher, #10010015), 49.6 ml

4. EDTA 10 mM

- EDTA (Thermo Fisher, #15575020), 500 mM, 1 ml
- *PBS (Thermo Fisher, #10010015), 49 ml

(*PBS should be Magnesium and Calcium free)

5. Tissue collection

- Collect the primary colorectal tumor tissue and the adjacent normal tissue in 50 ml falcon tubes containing 10 ml of Washing Medium (WM).
- Optimally, the volume of the collected tissue should be around 10 mm x 10 mm x 10 mm.
- The tissue can be stored up to 24 hours on ice in a fridge prior to processing.
- Thaw 1 ml Matrigel on ice (this amount is enough for 1 tumor and normal sample).
- Pre-warm the medium for organoid growth at 37 °C before use.

5.1 Normal and tumor organoid isolation

5.1.1. Washing

- Transfer the tissue to a new 50 ml falcon containing 20 ml of WM and vigorously shake to remove gut content.
- Repeat the previous step 1-2 times until the supernatant is clear.
- Transfer the tissue to a 10 cm dish.
- Using fresh sterile scalpels, cut the dissected tissue to 2-5 mm small pieces. And carefully remove fat and connective and necrotic tissues.
  - *For histology, choose 1-2 relatively big pieces and fix in 4% PFA and after 24 hours change to 70% ethanol and store at 4°C prior to paraffin embedding.*
  - *For DNA/RNA/protein collection, choose 3-5 random pieces and freeze them at -80°C.*
  - *Use remaining tissue "leftover" for the HIV test-PCR and other required diagnostic tests.*
- Collect the remaining pieces in a 50 ml falcon by adding 5 ml WM using 5 ml pipette.
- Spin at 800 rpm for 3 minutes at 4°C.

5.1.2. Incubation with EDTA 4 mM

- Remove the supernatant and add 10 ml of 4 mM EDTA and pipette up and down 4-5 times.
- Incubate for 30 min on ice.
- Pipet the pieces 15-20 times up and down with a 10 ml pipet to extract crypts/tumor cells.
- Repeat this step every 5 min.
- Let the pieces settle on ice and transfer the supernatant containing the crypts/tumor cells in a new 15 ml falcon.
- Spin the supernatant at 1400 rpm for 5 min at 4°C.

5.1.3. Incubation with EDTA 10 mM

- Add 10mM EDTA to the falcon containing the pieces. Repeat the step from 5.1.2.
- Combine both pellets from the 4 mM and 10 mM step, if needed.
- Spin down again at 1400 rpm for 5 min at 4°C. Remove the supernatant.

5.1.4. Organoid culture

- Resuspend the pellet in 150 µl medium and add 450 µl of Matrigel.
- Mix well using a 1 ml pipette tip and avoid air bubbles.
- In 6 wells of a 12-well plate make 4 x 15-20 µl drops of Matrigel (60-80 µl/well).
- Invert the plate and place it in the incubator for 20-30 min to allow the Matrigel to solidify.
- Add 1 ml of pre-warmed organoid medium containing Rho kinase inhibitor and Primocin.
  - *Only for the first 3 days add 1:1000 Rho kinase inhibitor to avoid anoikis (final concentration 10 µM).*
  - *Add 1:500 Primocin (Stock 50 mg/ml) until passage 3-4 to avoid contamination.*
- Change medium every 3-4 days.
- In general organoids can be split in 1:2 ratio after 1-2 weeks from the initial culturing.

6. Expanding and splitting organoid cultures

- Once organoid culture is confluent, remove the medium, add 1 ml of cold WM and resuspend the Matrigel drops using a 1000 µl pipette.
- Collect the organoids in a 5 ml Eppendorf tube (1-3 wells in 1 ml /Eppi).
- Mechanically shear the organoids using a 10 µl tip on top of a 1000 µl tip.
- Vigorously pipette up and down 20-30 times.
- Fill up with cold WM and spin down at 1200 rpm for 5 min at 4 °C.
- Remove the supernatant and resuspend the pellet in 75 % Matrigel.
- Seed into a 12-well suspension plate 4x15 µl drops of Matrigel/well (60 µl Matrigel/well).
- Invert the plate and place it in the incubator for 20-30 min to allow the Matrigel to solidify.
- Add 1 ml of the pre-warmed growing medium to each well.
- For the first 3 days add 1:1000 Y-27632 Rho kinase inhibitor (final concentration 10 µM).
- Change medium every 3-4 days.
- Organoids can be split in 1:3 or 1:4 ratio after 7-10 days.

7. Freezing and thawing of organoids

- Aspirate the medium, resuspend the Matrigel drops in 1 ml cold WM.
- Collect the organoids in a 5 ml Eppendorf tube (3 wells/ Eppi in 1 ml).

- Mechanically dissociate the organoids using a 10 µl tip on top of a 1000 µl tip.
- Vigorously pipette up and down 20-30 times.
- Add 5 ml cold WM and spin down at 1200 rpm for 5 min at 4 °C.
- Remove the supernatant and resuspend the cell pellet in 500 µl Recovery™ Cell Culture Freezing Medium/well.
- Place the cryovials in a freezing box at -80 °C.
- After one day, transfer the vials from -80 °C to a liquid nitrogen tank.
- When thawing frozen organoids, thaw the vial on ice and add 1 ml of cold WM.
- In a 5 ml Eppendorf tube with 4 ml of WM add the cells.
- Spin down at 1200 rpm for 5 min at 4 °C.
- Remove the supernatant and wash the pellet with 5 ml cold WM. Repeat the previous step.
- Remove the supernatant and resuspend the cell pellet in 75 % diluted Matrigel.
- Seed as indicated in ***6***. Typically, one cryovial is seeded in 1-2 wells of a 12- well plate.